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Gender-related differences in susceptibility of A/J mouse to benzo[*a*]pyrene-induced pulmonary and forestomach tumorigenesis

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Abstract

Benzo[*a*]pyrene (BP) is a suspected human carcinogen and is known to produce tumors in the lung and forestomach of mice. Glutathione (GSH) S-transferases (GST) play a major role in the detoxification of the ultimate carcinogen of BP, (+)-*anti*-7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene ((+)-*anti*-BPDE). Previous studies have shown gender-related differences in the expression of GST isoenzymes in mice. The present study was designed to test the hypothesis whether gender-related differences in the expression of GST isoenzymes can affect the susceptibility of mice to BP-induced lung and forestomach tumorigenesis. The expression of π class isoenzyme mGSTP1-1, which is highly efficient in the detoxification of (+)-*anti*-BPDE, was approximately 3.0- and 1.5-fold higher in the liver and forestomach of male A/J mouse, respectively, as compared with the female. The levels of other major GST isoenzymes, mGSTA3-3 (α class), mGSTM1-1 (μ class) and mGSTA4-4 (α class), were also significantly higher in the liver of the male mouse as compared with the female. While pulmonary mGSTP1-1 expression did not differ significantly between male and female A/J mice, the expression of mGSTA3-3, mGSTM1-1 and mGSTA4-4 was significantly higher (1.4–4.0-fold) in the lung of the male A/J mouse as compared with the female. At lower concentrations of BP (0.5 mg BP/mouse), the tumor incidence/multiplicity was significantly higher in the lung as well as in the forestomach of female mice as compared with male mice. For example, while 30% of the female mice developed pulmonary tumors 26 weeks after the first 0.5 mg BP administration, none of the male mice had tumors in their lungs. At higher doses of BP (1.5 mg BP/mouse), however, this differential was either abolished or relatively less pronounced. Our results suggest that up to a certain threshold of BP exposure the levels of GST isoenzymes may be an important determinant of susceptibility to BP-induced tumorigenesis in mice.

Keywords: Benzo[*a*]pyrene; Chemical carcinogenesis; Glutathione transferase; Detoxification; Cancer susceptibility

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), such as

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benzo[a]pyrene (BP), are well known carcinogens in animal models and their widespread presence in the environment poses a significant health hazard to humans. BP is by far the best studied PAH and is known to induce tumors in the lung, skin and forestomach of experimental mice [1,24,30]. It is well known that PAHs, including BP, require metabolic activation for the generation of their ultimate carcinogens, the diol epoxides [6,31]. For example, (+)-*anti*-7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene ((+)-*anti*-BPDE) is believed to be the ultimate carcinogenic metabolite of BP [2,29]. Even though several different mechanisms can convert PAH diol epoxides to less reactive species [4,32,33], the toxicologically most important metabolic pathway of these compounds is their glutathione (GSH) S-transferases (GST) catalyzed conjugation with GSH [5,11,13,20–22]. Mammalian GST isoenzymes can be classified into four major classes, i.e. α, μ, π and θ, on the basis of their physicochemical properties [7,15,16]. The GST isoenzymes of different classes are known to exhibit overlapping yet distinct substrate specificities [7,15]. For example, the π class human and rat GST isoenzyme is significantly more efficient than other classes of GSTs in catalyzing the GSH conjugation of (+)-*anti*-BPDE [20,21]. More recently, we have determined the relative contributions of various classes of murine GSTs in hepatic detoxification of (+)-*anti*-BPDE [10]. These studies reveal that the relative contribution of the π class isoenzyme (mGSTP1-1 according to the recently recommended nomenclature for murine GSTs) [7] far exceeds (about 79%) the combined contributions of other predominant hepatic isoenzymes, including α class mGSTA3-3, μ class mGSTM1-1 and α class mGSTA4-4. Despite significant advances toward metabolism of PAHs, the factors that determine the susceptibility to PAH-induced cancer are poorly defined.

Recent studies from our laboratory have shown that the female CD-1 mice are relatively more susceptible to BP-induced pulmonary tumorigenesis than the male mice [23]. In these studies, a single dose of 3 mg BP/mouse produced more tumors in the lungs of the female CD-1 mice as compared with the male mice. Based on these observations, we hypothesized that gender-related differences in the susceptibility of mice to BP-induced cancer may not be unique for the CD-1 strain or the lung tissue. In addition, we postulated that the gender-related differential in BP-induced cancer may be abolished at higher concentrations of the carcinogen because male mice having higher levels of mGSTP1-1 than the female would be able to detoxify (+)-*anti*-BPDE more efficiently only up to a certain threshold of BP exposure. During the present studies, we have systematically examined these hypotheses by quantitating GST isoenzymes in the liver (the main detoxification site) and target organs (lung and forestomach) of male and female A/J mice and by comparing the susceptibility of male and female A/J mice to BP-induced pulmonary and forestomach tumor incidence/multiplicity at two different doses of the carcinogen. The results of the present study indicate that while female A/J mice are relatively more susceptible than the male mice to BP-induced pulmonary as well as forestomach tumorigenesis at lower doses of BP, this differential is either abolished or relatively less pronounced at higher BP concentrations. The significance of these findings in relation to PAH exposure, GST isoenzyme expression and human health are discussed.

2. Materials and methods

2.1. *Benzo[a]pyrene-induced tumorigenesis experiments*

Male and female A/J mice (8 weeks old) were obtained from the National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD. The mice were divided into two groups of 10 mice for each sex. The mice were fed AIN-76 semipurified diet (ICN, Aurora, OH) for 1 week prior to the BP administration. The animals were given three oral administrations of the desired concentration of BP (0.5 or 1.5 mg BP/mouse suspended in 0.2 ml corn oil) every 14 days. The animals were killed by cervical dislocation 26 weeks after the first BP administration. The lung and forestomach tissues were collected and fixed in 10% buffered formalin and the tumor nodules were counted under a dissecting microscope.

2.2. *Purification and quantitation of GST isoenzymes*

The male and female A/J mice (8 weeks old) were fed AIN-76 semipurified diet for 1 week prior to the

collection of tissue samples. Hepatic, pulmonary and forestomach GST isoenzymes of the male and female A/J mice were quantitated by using a protocol described by us previously [9], which involves GSH affinity chromatography followed by reverse-phase HPLC. Briefly, equal amounts of liver, lung or forestomach tissues (0.25 g for liver and forestomach and 0.5 g for lung) from male and female A/J mice were homogenized in 10 mM potassium phosphate buffer (pH 7.0) containing 1.4 mM 2-mercaptoethanol. The homogenate was centrifuged at 14 000 × g for 40 min and the supernatant fraction was dialyzed against 22 mM potassium phosphate buffer (pH 7.0) containing 1.4 mM 2-mercaptoethanol. The dialyzed supernatant was subjected to GSH linked to epoxy-activated Sepharose 6B affinity chromatography to isolate total GSTs (mixture of isoenzymes). The GSH affinity chromatography was performed by the method of Simons and Vander Jagt [25], with some modifications described by us previously [26]. The GSTs retained on the affinity column were eluted by using equal volumes of the elution buffer (5 mM GSH in 50 mM Tris–HCl (pH 9.6) containing 1.4 mM 2-mercaptoethanol). Individual GST isoenzymes were separated and quantitated by reverse-phase HPLC analyses of equal volumes of affinity-purified GST preparations. A Waters Delta-Pak C_{18} reverse-phase column (150 × 3.9 mm) attached to a Waters HPLC system equipped with a Millennium 2010 chromatography manager and a Model 996 photodiode array detector was used for reverse-phase HPLC. The column was pre-equilibrated with 61% solvent I (5% acetonitrile/0.1% trifluoroacetic acid) and 39% solvent II (90% acetonitrile/0.1% trifluoroacetic acid). The GST subunits were eluted with a 30 min linear gradient of 39–69% solvent II at a column flow rate of 1 ml/min. The individual GST isoenzymes were identified on the basis of their elution profile during reverse-phase HPLC analysis and/or Western blot analysis as described by us previously [9,12]. The GST isoenzymes were quantitated by using a standard curve for corresponding isoenzymes.

3. Results

Previous studies have shown that three administrations, 2 weeks apart, of 2 mg BP/mouse results in 100% tumor incidence both in the lung and forestomach of female A/J mice [24,30]. To determine gender-related differences, if any, in the susceptibility of mice to BP-induced tumor incidence, we selected doses of 0.5 and 1.5 mg BP/mouse. As shown in Fig. 1, while 30% of the female mice developed pulmonary tumor nodules 26 weeks after the first 0.5 mg BP administration, none of the male mice had tumors in their lungs. As in the case of the lung, 40% of the female mice had forestomach tumors, whereas none of the males had forestomach tumors at a 0.5 mg BP concentration. While the gender-related differential in forestomach tumor incidence was completely abolished at higher concentrations of BP (1.5 mg BP/mouse), the BP-induced pulmonary tumor incidence was still higher in the female A/J mice (80%) as compared with the male (50%) even at 1.5 mg BP exposure.

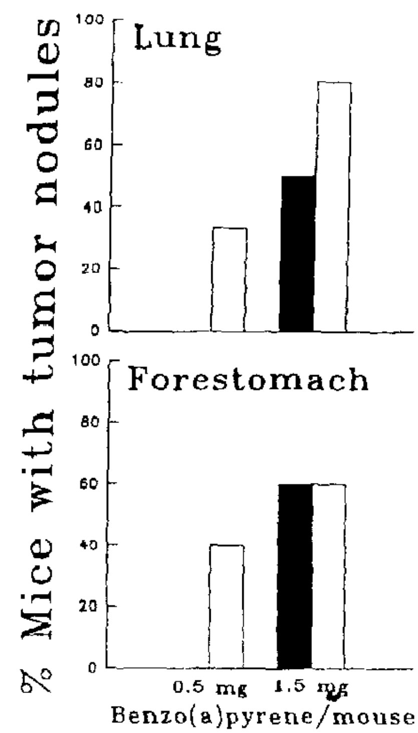

Fig. 1. Benzo[a]pyrene-induced pulmonary and forestomach tumor incidence in male (■) and female (□) A/J mice. $P>0.05$, male versus female at 1.5 mg BP/mouse dose by Student's t-test.

BP-induced pulmonary and forestomach tumor multiplicities (average number of tumor nodules/

Table 1

Benzo[a]pyrene-induced pulmonary and forestomach tumor multiplicity in male and female A/J mice

Sex	BP dose (mg BP/mouse)	Average number of tumor nodules/mouse	
		Forestomach	Lung
Male	0.5	0[a]	0[a]
Female		0.4 ± 0.2[b]	0.5 ± 0.3
Male	1.5	0.7 ± 0.2	1.5 ± 0.6
Female		1.2 ± 0.4[c]	2.4 ± 0.7[c]

[a]None of the mice in this group had detectable tumor nodules.
[b]Data represent the mean ± SE ($n = 10$).
[c]$P>0.05$, male versus female by Student's t-test.

mouse) were also compared in male and female A/J mice and the results are summarized in Table 1. As described above, there were no tumors either in the lung or forestomach of male A/J mice treated with 0.5 mg BP. At this dose of BP, the average numbers of tumor nodules/mouse in the lung and forestomach of female mice were 0.5 and 0.4, respectively. The gender-related differential in BP-induced tumor multiplicity was also evident at higher concentrations of BP exposure. For example, at 1.5 mg BP/mouse, the average number of tumor nodules/mouse in the lung of female mice was 2.4 as opposed to 1.5 observed in the lung of male mice. Likewise, the BP-induced forestomach tumor multiplicity was relatively higher in the female mice (1.2 tumor nodules/mouse) as compared with the male mice (0.7 tumor nodules/mouse). Taken together, these results suggest that BP elicits a differential tumorigenic response in male and female A/J mice and that this differential is relatively more prominent at lower doses of BP exposure.

The GST isoenzymes of the liver, lung and forestomach of male and female A/J mice were quantitated by using a protocol which involves GSH affinity chromatography and reverse-phase HPLC and the results are summarized in Table 2. Fig. 2 shows representative reverse-phase HPLC profiles of affinity-purified GST preparations from the liver of male and female A/J mice. The levels of minor hepatic GST subunits, labeled as M2, M3 and M4 in Fig. 2, are not included in Table 2 as collectively these isoenzymes account for less than 5% of the total GST protein in the liver of both male and female A/J mice. Significant quantitative differences were observed in GST isoenzyme expression between tissues of male and female A/J mice. For example, the mGSTP1-1 content was approximately 3.0-fold higher in the liver of male mice as compared with female mice. The levels of other major hepatic GST isoenzymes were also significantly higher (2.2–3.0-fold) in the male than in the female mice. The gender-related differences in GST isoenzyme expression were relatively less pronounced in the target organs (lung and forestomach) than in the liver. In the forestomach, the mGSTP1-1 content was higher by about 1.5-fold in the males as compared with the females ($P < 0.05$ by Student's t-test). However, the difference in the forestomach content of mGSTA4-4 was relatively more pronounced than mGSTP1-1 between males and females (male to female ratio 1.9). While the mGSTP1-1 content did not differ significantly between the lung of male and female mice, the constitutive expression of other pulmonary GST isoenzymes was significantly higher (1.4–4.0-fold) in male mice as compared with female mice. Collectively the data presented in Table 2 clearly demonstrate gender-related quantitative differences in constitutive expression of GST iso-

Table 2

Relative abundance of the predominant GST isoenzymes in the liver, lung and forestomach of male and female A/J mice

Tissue	GST isoenzyme	GST isoenzyme content (μg/g wet tissue)		
		Male	Female	Ratio (male/female)
Liver	mGSTA3-3	589 ± 27[a,b]	304 ± 33	1.9
	mGSTP1-1	512 ± 72[b]	169 ± 59	3.0
	mGSTM1-1	586 ± 57[b]	266 ± 35	2.2
	mGSTA4-4	32 ± 7[b]	14 ± 2	2.3
Forestomach	mGSTA1-2	24 ± 6	23 ± 7	1.0
	mGSTP1-1	172 ± 33[b]	114 ± 2	1.5
	mGSTM1-1	235 ± 52	280 ± 16	0.8
	mGSTA4-4	115 ± 9[b]	62 ± 19	1.9
Lung	mGSTA3-3	32 ± 3[b]	23 ± 3	1.4
	mGSTP1-1	53 ± 7	47 ± 3	1.1
	mGSTM1-1	85 ± 8[b]	53 ± 4	1.6
	mGSTA4-4	24 ± 3[b]	6 ± 1	4.0

[a]Data are the mean ± SD of four determinations. For the quantitation of hepatic GST isoenzymes, the tissues from an individual mouse were used. For the quantitation of forestomach and pulmonary GST isoenzymes, tissue samples from two mice were pooled.
[b]Significantly different from female, $P < 0.05$ by Student's t-test.

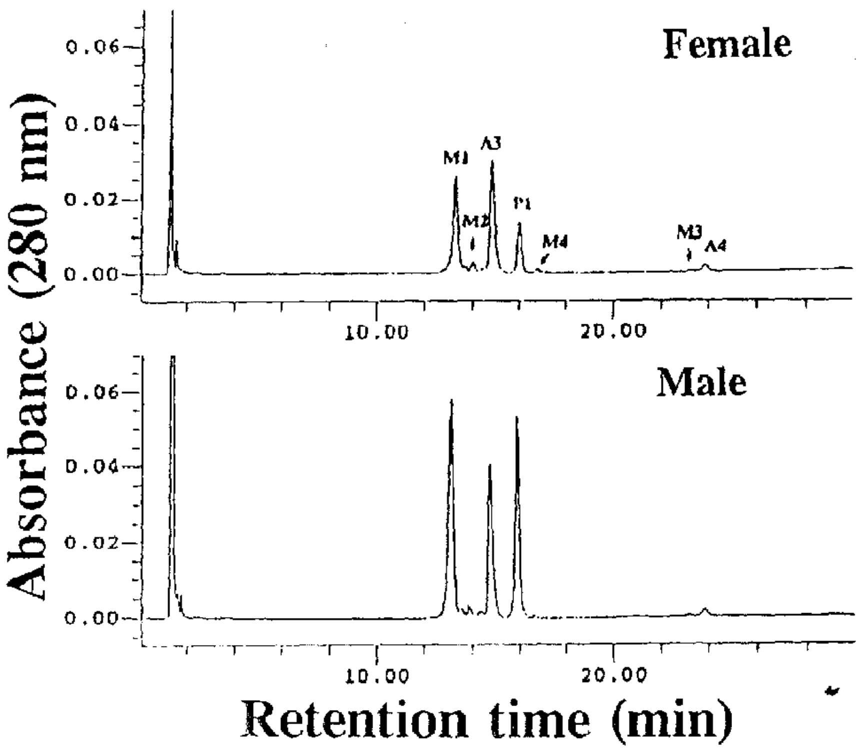

Fig. 2. Representative reverse-phase HPLC elution profiles of GSH affinity-purified GSTs from equal amounts (0.25 g) of the liver of male and female A/J mice.

enzymes in the liver as well as target organs of A/J mice.

4. Discussion

Previous studies from our laboratory have shown that a single administration of 3 mg BP/mouse causes relatively more tumors in the lungs of female CD-1 mice than in the male [23]. The results of the present study reveal that gender-related differences in the susceptibility to BP-induced cancer in mice are not confined to the CD-1 strain or to the lung tissue. Both tumor incidence and tumor multiplicity data clearly reveal that BP is more tumorigenic in the lung as well as the forestomach of female A/J mice as compared to the male. The gender-related differential in BP-induced tumor incidence/multiplicity is relatively more pronounced at lower levels of BP exposure. At higher concentrations of the carcinogen (1.5 mg BP/mouse), this gender-related differential in the carcinogenicity of BP is markedly attenuated as indicated by a similar tumor incidence in the forestomach of both male and female A/J mice. However, even at a dose of 1.5 mg BP, the differential response of this carcinogen is reflected by a relatively higher tumor incidence in the lung and higher tumor multiplicity in the lung as well as in the forestomach of female mice. It is possible that at doses of BP higher than 1.5 mg/mouse, the differential tumorigenic response of BP to male and female mice may be completely abolished. This may be because the higher concentrations of BP may overcome the differential carcinogenic response of the carcinogen. In particular, if the mechanisms which protect against BP-induced carcinogenicity are more efficient in male mice than in their female counterparts, as reflected in their GST isozyme profile, an overload of the carcinogen beyond the threshold of the protective capacity of male mice would make both males and females equally susceptible to BP-

induced cancer. The results presented in this study seem to be consistent with this postulate.

While the mechanisms responsible for the differential carcinogenicity of BP to male and female A/J mice are not completely understood, our results suggest that the differences in GST compositions of male and female mice may be an important determinant of the sex-related response of BP. In the liver of male mice, each GST isoenzyme is clearly more abundant as compared with the female. More prominently, mGSTP1-1, which has been shown to be the major GST isozyme responsible for the conjugation of (+)-*anti*-BPDE in the liver of A/J mice [10], is about 3.0-fold higher in the male liver as compared with the female. Other major hepatic GST isoenzymes of the α and μ classes, which also contribute to some extent to the GST catalyzed conjugation of (+)-*anti*-BPDE with GSH, are also more abundant in the liver of male mice. These results suggest that the male A/J mice may be relatively more efficient than the female in detoxifying (+)-*anti*-BPDE because the liver is the main site of xenobiotic biotransformation. Thus, it is reasonable to speculate that the levels of (+)-*anti*-BPDE in the circulation of the male mice would be less than in the female mice and that at low levels of BP exposure the male mice would be better protected against the carcinogenic effects of BP than the female A/J mice. The results presented in this study support this contention. The relative abundance of certain GST subunits is also significantly higher in the lung and forestomach of male mice as compared with female mice. In particular, the mGSTP1-1 content in the forestomach of male mice is significantly higher relative to the female. An even more prominent higher abundance of an α class GST (mGSTA4-4) is observed in the lung as well as in the forestomach of male mice (4.0- and 1.9-fold, respectively). These results suggest that in addition to the liver, the target organs in male mice may also be more efficient in the detoxification of (+)-*anti*-BPDE. Therefore, it is highly likely that the gender-related differences in the susceptibility of A/J mice to BP-induced pulmonary and forestomach tumorigenesis may, at least in part, also be due to differences in GST isoenzyme profiles of the target organs.

While it is not possible to extrapolate these results with mice to humans, it is important to note that sex-related qualitative and quantitative differences in the expression of GST isoenzymes have been documented in human tissues [27,28]. Furthermore, it has been reported that the risk of lung cancer is relatively higher in women than in men per cumulative dose of cigarette smoking [18,19]. Also, there is limited evidence that women have a higher susceptibility to tobacco smoke carcinogens than men [17]. More recently, it has been shown that within the *P53* tumor suppressor gene, *anti*-BPDE preferentially modifies guanine residues in the same mutational hotspots that are frequent in human cancers [3]. While some of these mutational hotspots are common to various malignancies, one affecting codon 157 of p53 is characteristic for lung cancer [8,14]. Since *anti*-BPDE is derived from cigarette smoke, this study provides a partial mechanistic explanation for the well established epidemiological correlation between smoking and lung cancer. Since PAHs, including BP, are abundant in our environment, the possibility that sex-related differences in the susceptibility of humans to tobacco carcinogens is a result, at least in part, of differential expression of GST isoenzymes cannot be ignored. Thus, further studies are needed to determine if gender-related differences in GST isoenzyme expression are a determinant of differential susceptibility of humans to PAH-induced cancer.

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